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ORIGINAL ARTICLE

Prokaryotic Expression of Antimicrobial Peptide CATH PR1–2 from the Skin of *Paa robertingeri* in *Escherichia coli*

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Abstract The aim of this study was to investigate the prokaryotic expression of antimicrobial peptide cathelicidin (CATH) PR1 and PR2 from the skin of *Paa robertingeri* in *Escherichia coli*. Two active peptides, CATH PR1 and CATH PR2, belong to the CATH family in the skin of *P. robertingeri*. CATH PR1 has a relatively high antimicrobial activity, especially for the drug-resistant strains found in clinical practice; however, no antimicrobial activity has been found in CATH PR2. The molecular weights of both CATH PR1 and CATH PR2 are relatively low (3195.88 and 2838.34 Da, respectively). Thus, the genetic processes, as well as the expression and purification of these proteins, are difficult to perform. Therefore, in this study, CATH PR1 and CATH PR2 genes were tandem ligated and then connected to the plasmid pET-32a. This reconstructed plasmid was then transfected into the expression vector *E. coli* BL21 to construct the recombinant expression system. The fusion expression of peptide PR was stable in *E. coli* after induction with 1.0 mol/L isopropyl β-D-1-thiogalactopyranoside at 37°C for 4 h. The antimicrobial activity assay using *Staphylococcus aureus* (Song) and *Candida albicans* 08030102 showed that the antimicrobial activity of PR was similar to the antimicrobial activity of CATH PR1. This study showed that artificial modification of the amino acid sequences of PR1 and PR2 could result in better protein expression in prokaryotes, and the fusion protein expressed had relatively high antimicrobial and other biological activities. In conclusion, the findings suggest future prospects of the commercialization of this method.

Keywords *E. coli* BL21, fusion expression, *Paa robertingeri*, recombinant protein PR

1. Introduction

The antimicrobial peptides in the cathelicidin (CATH) family consist of an N-terminal signal peptide domain, a conservative intermediate CATH domain, and a C-terminal mature peptide domain. The mature peptide plays an important role in the innate immune system of most vertebrates (Brandenburg *et al*., 2012; Ling *et al*., 2013; Conlon and Mechkarska, 2014). Previously, the total RNA was extracted from the skin of *Paa robertingeri* to build the cDNA library of the skin. The primers were designed according to the features of the sequences of CATH family genes, and two cDNA encoding the precursors of CATHs were obtained by semi-nested polymerase chain reaction (PCR). Alignment analysis deduced the sequences of two mature peptides, named as CATH PR1 and CATH PR2. The mature peptide of CATH PR1 consists of 29 amino acid residues (RKCN LFCKAKQKLKSLSSVIGTVVHPPRG), and the mature peptide of CATH PR2 consists of 25 amino acid residues (KECKDYLCKLLMKLGSSSHIESIDP) (Yin, 2015).

Antimicrobial activity assays have demonstrated that CATH PR1 has remarkable broad-spectrum antimicrobial activities. Thirteen drug-resistant strains were assayed, and CATH PR1 showed antimicrobial activities against *Pseudomonas maltophilia* 7404, *Bacillus cereus* (Song), *B. subtilis* (Guizhou), *Candida albicans* 08030102, and *C. glabrata* 08A802, with the minimal inhibitory concentration (MIC) of 75, 37.5, 37.5, 37.5 and 37.5 μg/mL, respectively; especially for bacteria commonly found in trauma-related infections such as *Staphylococcus*

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aureus (Song), the MIC was only 37.5 μg/mL. However, no antimicrobial activity against these strains was found in the case of CATH PR2 (Yin, 2015). Chemical synthesis of antimicrobial peptides involves high costs, while manual extraction of antimicrobial peptides is associated with great wastage and damage to resources. With the emergence of multiple protein induction and expression systems, the prokaryotic expression systems represented by *E. coli* have significantly improved. The *E. coli* expression system has several advantages including rapid growth, low cost, and high expression. However, the molecular weight of CATH PR1 is very low. As a result, this molecule can be easily decomposed by host cells. In addition, this peptide is toxic for the host bacteria and thus cannot be expressed directly. Therefore, fusion expression is required to obtain a large amount of this antimicrobial peptide. At present, there has been successfully expressed Human beta-defense-6 (Ma, 2010), LL–37 (Li *et al*., 2006), fusion protein hEGF- AWRK6 (Zhang, 2014).

The molecular weights of both CATH PR1 and CATH PR2 are relatively low (3195.88 Da and 2838.34 Da, respectively). Thus, the genetic processes, as well as the expression and purification of these proteins, are difficult to perform. Therefore, in the present study, CATH PR1 and CATH PR2 genes were tandem ligated and cloned to the *E. coli* expression vector pET-32a. Then, the cloned PR-pET32a was used to transfect the expression vector *E. coli* BL21. The activities of the expressed proteins were evaluated after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction.

2. Materials and Methods

2.1 Strains and plasmids Competent *E. coli* DH5α, *E. coli* BL21(DE3), plasmid pET-32a, CATH PR1-2 containing reconstructed plasmid pUC57s, *S. aureus* (Song), and *C. albicans* 08030102 were used in this study.

2.2 Equipment and reagents The equipment used in this study included the following: Techne PCR instrument (TC-4000), agarose gel electrophoresis apparatus (DYY-6C), vertical electrophoresis apparatus (Beijing Liuyi P4), Galanz microwave oven (G70D20CN1P-D2(S0)), microcentrifuge (TOMOS 1-14), high-speed refrigerated centrifuge (3-18R), ultrasonic cell disruptor (BILON-650V), electrically heated thermostatic water bath (HWS-26), ultraviolet spectrophotometer (INESA 752N), thermostatic shaking incubator (ZC-100B), electro-heating standing-temperature cultivator (SHP-500), and Eppendorf pipettes.

The following reagents were used in this study: 2 × Super Pfu PCR Master Mix, Biowest regular agarose, Tris-acetate-EDTA, T4 DNA ligase, 10 × T4 ligation buffer, IPTG, ampicillin, agarose, glucose, yeast extract powder, Bacto Peptone, acrylamide, SDS, ammonium persulfate, and tetramethylethylene diamine.

2.3 Expression vector construction

Amplification of the target sequences Enzyme digestion sites *Kpn*I (GGTACC) and *Hin*dIII (AAGCTT) are shown in italics with underline; the formic acid cutting locus (GACCCG) is shown in bold. They were added before and after the cDNA of the mature peptide of CATH PR1–2 to obtain the following sequences. The cDNA sequences of the mature peptide of CATH PR1–2 are displayed in parentheses.

CGG*GGTACC***GACCCG(**cggaagtgtaacttgttctgcaaag cgaagcagaagctgaaatctctgagctccgtcatcgggacggtcgttcatcca cctcgaggaaaagaatgcaaagattattcgtgtaaactgcttatgaaacttgga tcctccagccacatcgaaagcatcgatccc) *AAGCTT*

The CATH PR1–2 gene was synthesized and then loaded to the vector pUC57s.

The primers designed were as follows:

PR1-2F-*Kpn*I: CGGGGTACCGACCCGCGGAAGTGTA ACTTG

M13-R: AGCGGATAACAATTTCACACAGG

The plasmid with the synthesized gene was used as the template. PR1-2F-*Kpn*I/M13-R and 2 × Super Pfu PCR Master Mix (Hangzhou Bao Sai Biological Company, Hangzhou, China) were used for PCR amplification to obtain the target sequences. The conditions for PCR amplification were as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 68°C for 10s for 30 cycles, followed by 68°C for 5 min. Then, the products were cooled at 10°C for 2 min. Agarose gel electrophoresis (1%) was used for detection.

Dual-enzyme digestion The PCR products obtained were collected using a rapid PCR product purification kit (Hangzhou Bao Sai Biological Company), and then *Kpn*I/*Hin*dIII (Takara) was used for dual-enzyme digestion; dual-enzyme digestion of the pET-32a plasmid was also performed with *Kpn*I/*Hin*dIII (Takara). The mixture was first placed at 37°C overnight for enzyme digestion then placed at 37°C for 2 h enzyme digestion. The products obtained by dual-enzyme digestion were measured by agarose gel electrophoresis.

Connection and transfection After collecting the products of dual-enzyme digestion using the purification kit, T4 DNA ligase was used for the connection at 4°C overnight. The system used for the connection was as

follows (Table 1). The PR-pET-32a after the connection was transfected into the competent DH5α cells.

Table 1 The reagents and volume of the connection system.

Reagents	Volume
Product from the target vector	2 μL
Target sequence	6 μL
10× T4 DNA ligase	1 μL
T4 DNA ligase	1 μL
Total	10 μL

The competent DH5α cells were thawed on ice. The products of the connection were mixed evenly with the competent cells. They were placed on ice for 30 min and then at 42°C for 60 s, after which the mixture was immediately immersed in ice for 2 min. Luria-Bertani (LB) culture medium (1000 μL) was then added, and the mixture was shaken for 1 h at a frequency of 150 rpm. The mixture was then centrifuged at 12,000 r/min at room temperature for 4–5 min to obtain a supernatant of 500 μL, which was discarded. The residual mixture was mixed, and then 200 μL of the mixture was applied evenly on the LB (A^+) solid culture medium containing ampicillin, which was inversely placed into the incubator and cultured overnight.

The bacterial clones were selected and identified by PCR using 2 × Taq PCR mix (Hangzhou Bao Sai Biological Company), T7/T7ter, and primers. The positive clones were sent to Nanjing Jinsirui Biotechnology Co., Ltd., Nanjing, China, for sequencing.

2.4 Fusion protein expression If the inserted segments were correct, the positive clones were transfected into the competent *E. coli* BL21 (DE3) cells, and the monoclones of the strains were selected for further replication and induction of expression. The strains were stored in glycerinum at –80°C.

The strains were cultured in LB (A^+) liquid culture medium containing ampicillin at 37°C with shaking until the OD_{600} was 0.6–1.0. IPTG was then added to obtain a final concentration of 1.0mM to induce the expression for 4 h. Strains without IPTG induction were also cultured as control. The cells were then centrifuged at 12 000 r/min at room temperature for 5 min to collect the bacteria, and Tris-HCl (pH 7.0) was used to resuspend the bacteria. SDS-polyacrylamide gel electrophoresis (PAGE) (15%) was used to evaluate whether the expression of the fusion protein was successful. The resuspended bacteria were subjected to ultrasonication for 1 min (working for 3 s followed by resting for 3 s), followed by centrifugation at 12,000 rpm and 4°C for 5 min to collect the supernatant for SDS-PAGE (15%).

2.5 Antimicrobial activity assay LB liquid medium was used to dilute the *S. aureus* and *C. albicans* to obtain the concentration of 10^{-3}, and then applied evenly on the surface of the LB solid medium. The sterile water was used to dilute the protein sample. A sterilized Oxford cup was placed at the center of the culture medium; 20 μL of the protein samples to be tested were added into the Oxford cup and cultured at 37°C overnight to examine for the presence of inhibition zones.

3. Results

3.1 Expression vector construction Figure 1 shows the results of the amplification of the pUC57s vector using primers for PR1-2F-*Kpn*I/M13-R, and Figure 2 shows the results of the dual-enzyme digestion of the segments obtained using *Kpn*I/*Hin*dIII (Takara). The size of the target sequences was consistent with the theoretical size (178 bp).

3.2 Connect product and the result of sequencing analysis After connecting the segments from the dual-enzyme digestion with the plasmid pET-32a, a single bacterial clone was selected for PCR detection, and the primers used were T7/T7ter that is universally used for

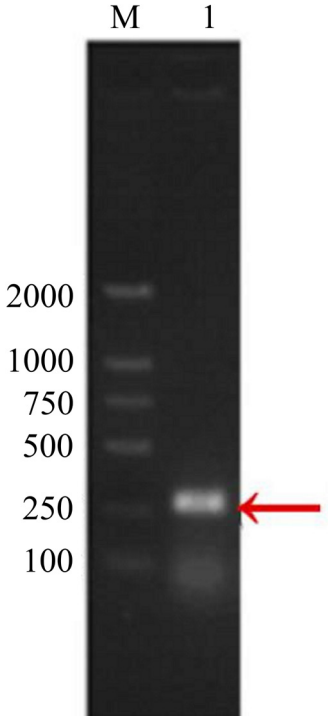

Figure 1 Electrophoresis of the PCR products. The arrow shows the PR segment obtained from the PCR amplification.

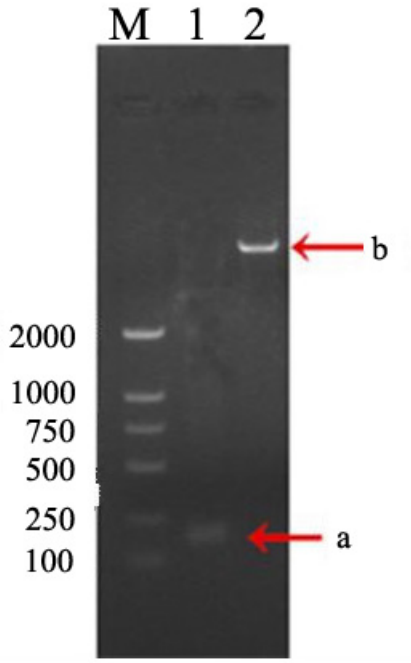

Figure 2 Electrophoresis of the PR and pET-32a. M- DNA marker; a- product from dual-enzyme digestion of the PR; and b- product from dual-enzyme digestion of the pET-32a.

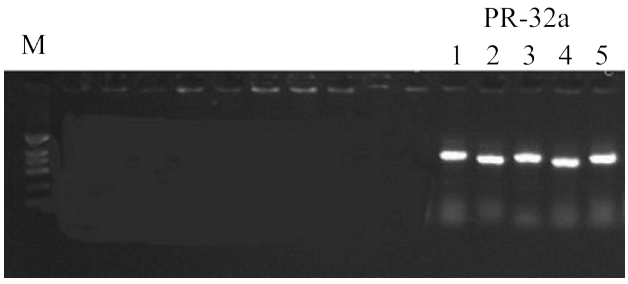

Figure 3 Electrophoresis of the PCR products of the bacterial clone. The red box shows the PCR products after amplification with the primers T7/T7ter.

plasmid pET-32a. The results are shown in Figure 3.

The sequencing results were analyzed by the DNAstar software, and the results are shown in Figure 4. The BioEdit software was used to compare the nucleotide sequences of the PR-32a and CATH PR1–2 (Figure 5). The results showed that the nucleotide sequences of PR-32a and CATH PR1–2 were highly consistent, suggesting that PR1–2 was correctly connected to the plasmid pET-32a. The box in Figure 6 shows the amino acid sequences after CATH PR1 was connected with CATH PR2. After the comparison, the results (Figure 7) showed that the amino acid sequences were highly consistent, suggesting that the reconstructed PR protein was correctly fused with pET-32a and expressed.

3.3 Expression of PR-32a fusion protein The bacteria were collected after the induction of expression and centrifuged to collect the supernatant for SDS-PAGE electrophoresis. The results (Figure 8) showed that the size of the protein expressed was consistent with the theoretical protein size (24189.76 Da), suggesting that the fusion protein was successfully expressed.

3.4 Antimicrobial activity assay The antimicrobial activity assay was used for the polypeptide samples, and the results showed that the fusion protein PR had antimicrobial effects against *S. aureus* and *C. albicans* (Figure 9).

4. Discussion

In genetic engineering studies, expression of the exogenous genes in the vector plays a dominant role in the consequent functional studies and applications of the recombinant proteins. The vectors commonly used in the prokaryotic expression system include glutathione transferase (GST), small ubiquitin-like modifier (SUMO), and pET systems (Ma *et al*., 2011), while the major expression host is the *E. coli* expression system. When fusing GST with the protein tag to express the antimicrobial peptide, the molecular weight of the antimicrobial peptide (< 10 kDa) is lower compared with that of the GST-fused protein (27 kDa). Thus, the percentage of the antimicrobial peptide in the fusion protein is relatively low (Li, 2009). Some other studies found that the expression of the protein after fusion of the antimicrobial peptide with the GST tag could induce the death of the host *E. coli*, which could be associated with the role of the fusion protein in killing prokaryotic cells (Hu *et al*., 1999). The SUMO expression system could connect the SUMO gene with the gene of the target protein to form a fusion expression vector for the expression. The SUMO fusion tag has a hydrophobic core and a hydrophilic surface, which could substantially increase the solubility and expression of the protein (Malakhov, 2004). Currently, the *E. coli* SUMO expression has been successfully used to express bacteriocin Lactin Q, cecropin ABP-CM4, human defensin-4, and krait CATH-BF, and the expression level was 110, 48, 166, and 22 mg/L, respectively (Ma *et al*., 2011; Li *et al*., 2010a, 2010b; Luan *et al*., 2014). However, the SUMO expression system is a novelexpression system developed recently, and a research team in the Emory University (GA, USA) acquired the patent of using the SUMO

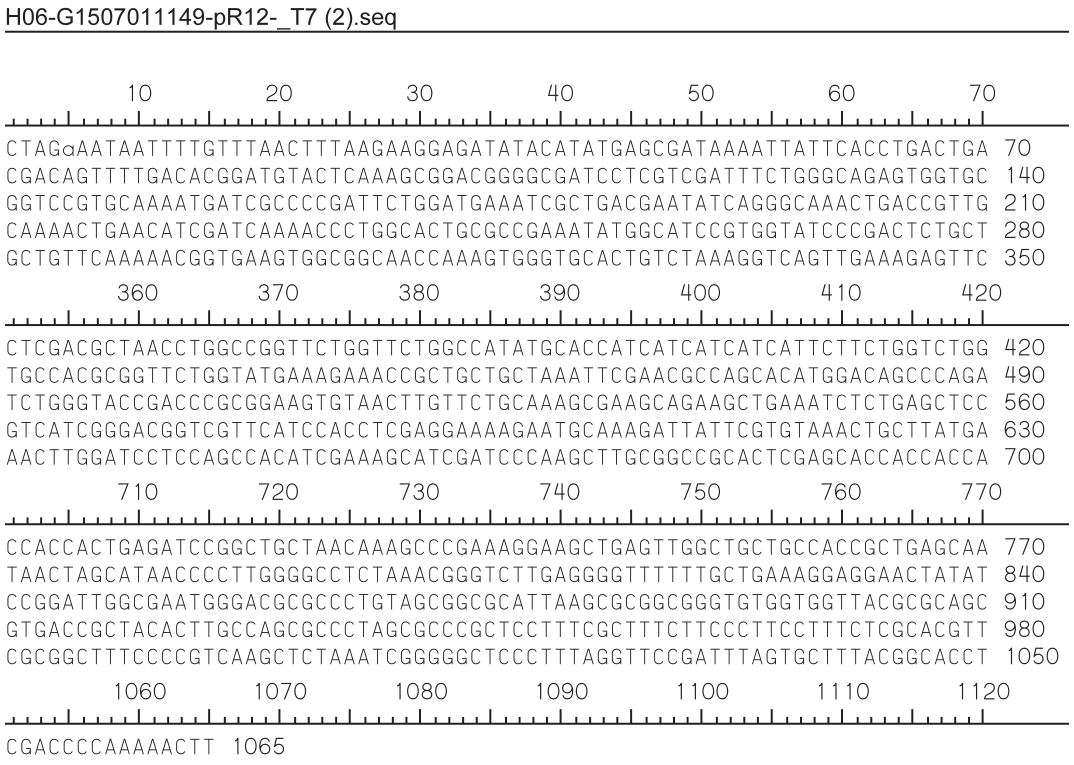

Figure 4 Nucleotide sequences displayed in the sequencing. The black box shows the sequences after PR1 and PR2 were connected.

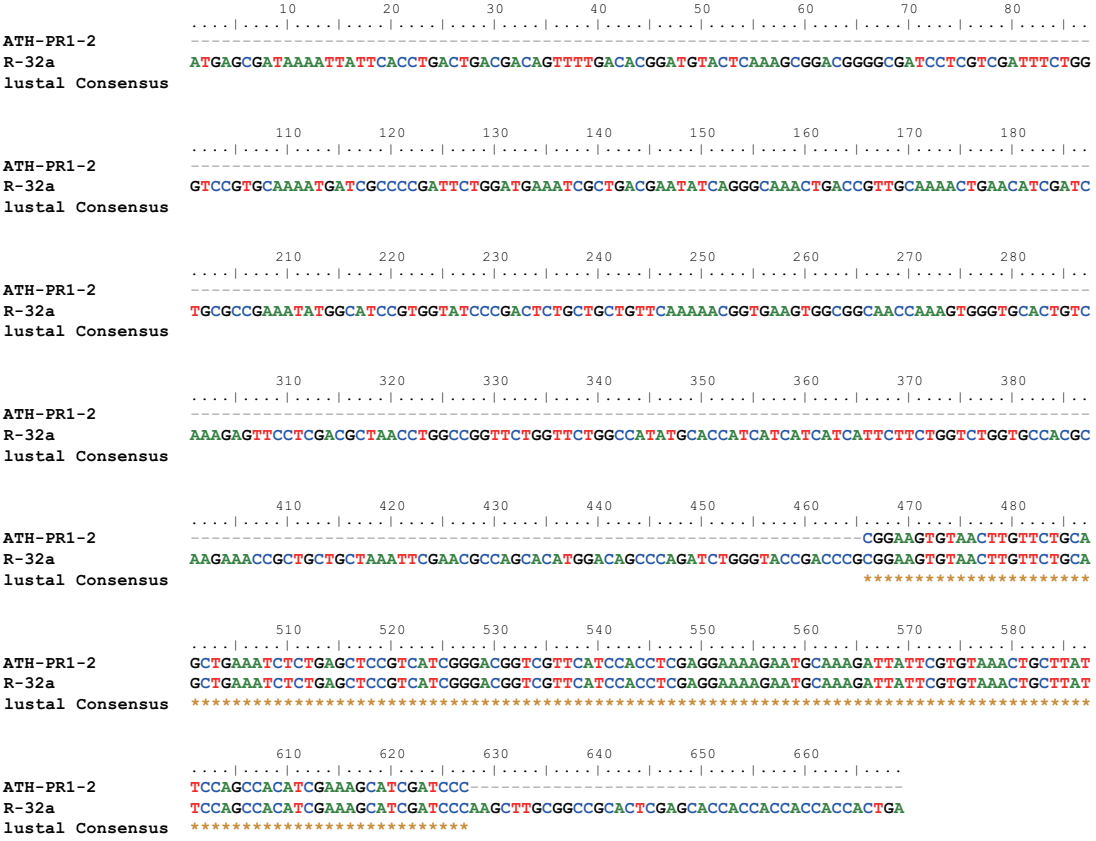

Figure 5 Comparison of the nucleotide sequences between PR-32a and CATH PR1–2. *Indicates the consistent nucleotide sequences; - indicates the sequences generated by the software.

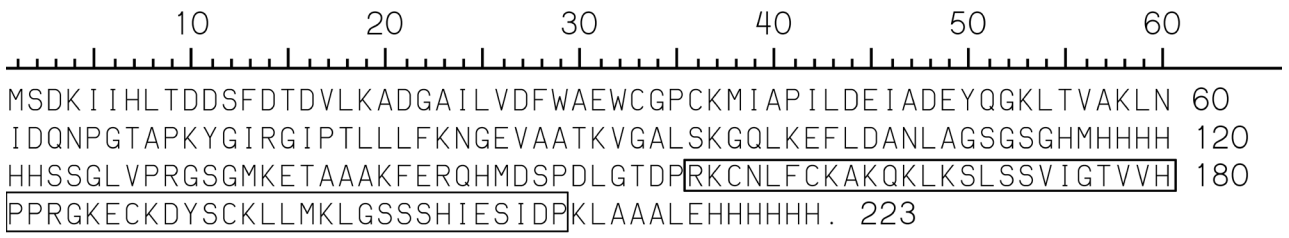

Figure 6 Amino acid sequences after PR1 and PR2 were connected.

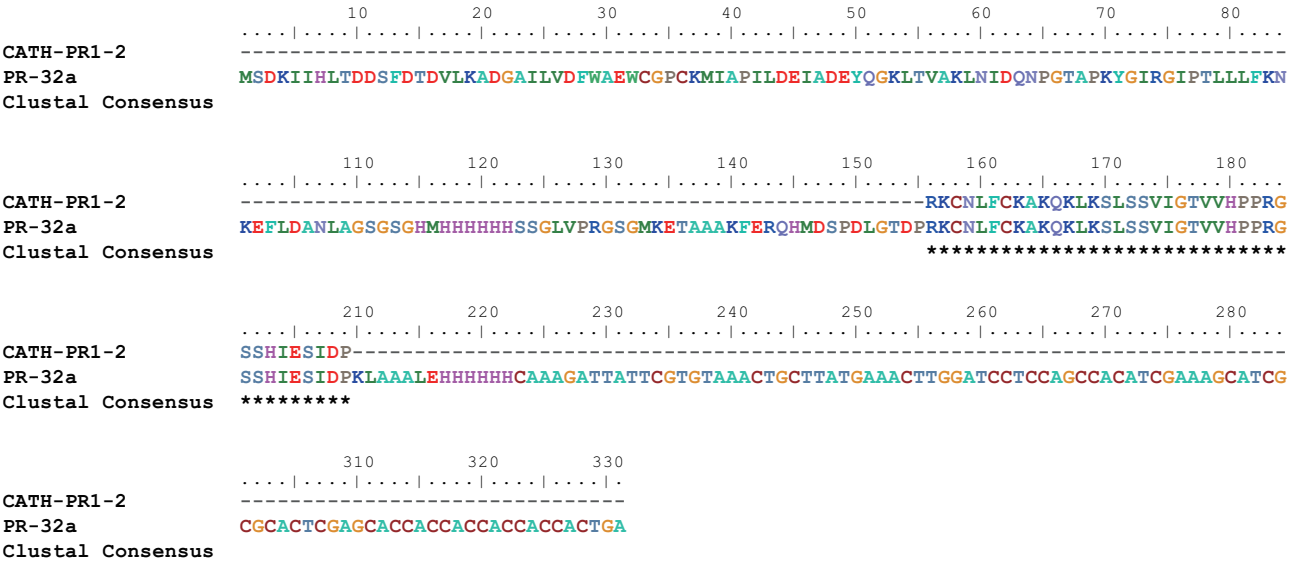

Figure 7 Comparison of the amino acid sequences between PR-32a and CATH PR1–2.*Indicates the consistent amino acid sequences; - indicates the sequences generated by the software.

expression system to obtain antimicrobial peptides in 2008 (Zhang *et al*., 2014). Such studies in China are very rare, and the experience and technologies related to this expression system are still lacking. The pET system is the most powerful system among the *E. coli* expression systems in expressing the recombinant proteins. The His-Tag in the pET vectors could facilitate the consequent protein separation and purification, while the Trx-Tag in the pET32a could promote the solubility and activities of the target proteins. Currently, this system has been successfully used to express human LL-37, fusion peptide hEGF-AWRK6, and quail Cc-CATHs in *E. coli* (Feng *et al*., 2011; Li *et al*., 2006; Zhang, 2014). Therefore, the pET-32a system was chosen as the prokaryotic expression vector for the expression of the exogenous protein of *P. robertingeri.*

The CATH family is a very important family of antimicrobial peptides from vertebrates, which possess broad-spectrum and more effective antimicrobial activities, as well as lower hemolytic activities and cytotoxicity compared with other families of antimicrobial peptides (Kosciuczuk *et al*., 2012; Wuerth and Hancock, 2011). CATHs were first identified in mammals, birds, and fishes; they have been recently discovered in amphibians. The first CATH family member, CATH-AL, was identified in *Amolops soloensis* in 2012, followed in *Limnonectes fragilis* and *P. robertingeri* (Yin, 2015). These findings demonstrated that the antimicrobial peptides in the CATH family play an important role in the innate immunity in vertebrates (Brand *et al*., 2013; Jorge *et al*., 2014; Saemann *et al*., 2007; Wang *et al*., 2015). Previous studies have shown that in addition to the potential antimicrobial effects, the antimicrobial peptides in the CATH family also prevent tissue damage and improve cell chemotaxis, wound repair, and angiogenesis (Brown *et al*., 2014; Wuerth and Hancock, 2011). For instance, LL-37 could bind to and neutralize the cytosolic DNA in keratinocytes, and thus reduce the damage caused by psoriasis in the body (Dombrowski *et al*., 2011; Wuerth and Hancock,

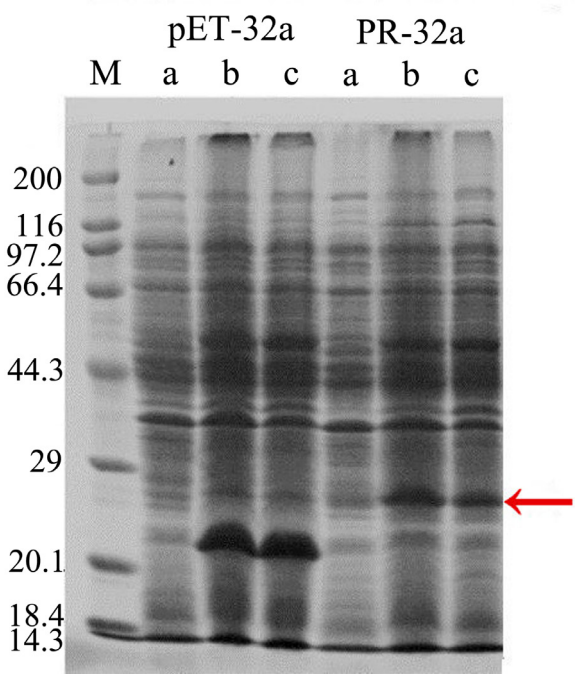

Figure 8 SDS-PAGE electrophoresis at 37°C, 1.0mM for 4 h. a- Not induced; b- whole-cell lysate proteins; and c- supernatant. The arrow shows the target protein.

2011). The precursors of the antimicrobial peptides in the CATH family consist of an N-terminal signal peptide domain, a conservative intermediate CATH domain, and a C-terminal mature peptide domain (Ma, 2010). The differences in the biological functions of these antimicrobial peptides are associated with the differences in the structures and the amino acid sequences in the C-terminal mature peptide domain.

Previous studies have shown that most antimicrobial peptides in the CATH family consist of α-helix. The amino acid sequences and the structures of the antimicrobial peptides could affect the antimicrobial activities. The amphiphilic α-helix could rapidly damage the cellular membrane and form transmembrane channels without toxic effects on the mammalian cells (Wuerth and Hancock, 2011; Zhang *et al*., 2010). The polypeptides carrying positive charges could interact with the bacterial membrane carrying negative charges via electrostatic interaction. Increasing the positive charges on the polypeptide molecules could enhance the antimicrobial effects (Yi *et al*., 2014). Previous studies have found that both PR1 and PR2 have the α-helical structure, while the net charge is +7 and 0 for PR1 and PR2, respectively. Therefore, it is speculated that the relatively strong antimicrobial activities of PR1 and the lack of antimicrobial activity of PR2 could be associated with the fact that PR2 does not carry any charge. However, PR2 may still have some other biological functions such as growth factor-like effects, besides improving repair. This speculation needs to be investigated further.

The molecular weight of PR is estimated to be 5990.1 Da using the online ProParam tool (http://web.expasy.org/protparam/). The theoretic isoelectric point is 9.59, the net charge is +7, and it has 4 residues carrying negative charges and 11 residues carrying positive charges. The UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/), an online protein analysis website, was used to predict the secondary structure of the recombinant PR peptide, and the results are as Figure 10.

The PR had an α-helical structure at the amino acids 4–20 and 34–43, while the others were coils. Ling *et al*. (2013) showed that for PR1, the amino acids from 3 to 21 formed an α-helix, while the others were random coils; for PR2, the amino acids from 2 to 14 formed an α-helix, while the others were random coils. Circular dichroism showed that the fusion peptide expressed after induction also had the α-helical structure, and all the seven positive charges were in the PR1 domain, suggesting that the experiment and the methods used were successful. The changes in the length and position of the α-helix in the PR are caused by the interactions between the two amino acid segments and the two molecules of PR1 and PR2 after the connection. The antimicrobial activity assay showed that the fusion peptide PR of *P. robertingeri* expressed after induction had evident antimicrobial effects, which could be caused by the maintenance of the α-helical structure and the positive charges (+7) on the synthesized peptide.

The present study showed that the artificial modification of the amino acid sequences of PR1 and PR2 could result in better protein expression in prokaryotes, and the fusion protein expressed had relatively high antimicrobial and other biological activities. The findings suggest future prospects of the commercialization of this method.

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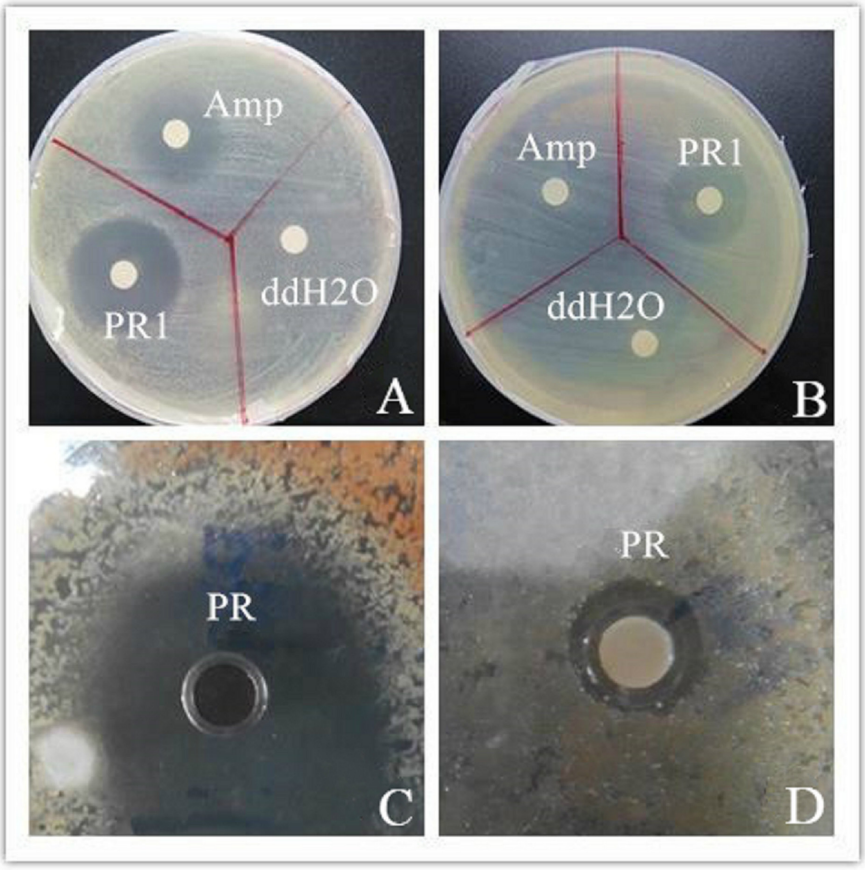

Figure 9 Results of the antimicrobial activity assay. A and C show the antimicrobial activity of the chemically synthesized PR1 and the fused protein PR against *Staphylococcus aureus* (Song); B and D show the antimicrobial activity of the chemically synthesized PR1 and the fused protein PR against *Candida albicans*.

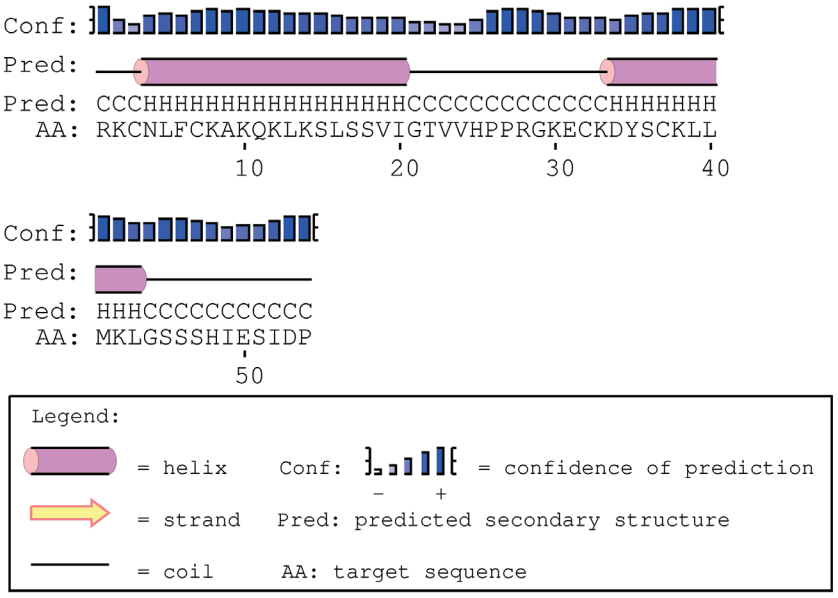

Figure 10 The secondary structure figure of the recombinant PR peptide.

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